Epidermal growth factor promotes stromal cells migration and invasion via up-regulation of hyaluronate synthase 2 and hyaluronan in endometriosis

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Objective: To investigate the role(s) of hyaluronan synthase 2 (HAS2) and hyaluronan in disease progression of endometriosis and epidermal growth factor (EGF)–induced motility changes of endometriotic cells.

Design: A case-control experimental study and in vitro primary cell culture study.

Setting: University hospital–affiliated research centers.

Patients: A total of 21 women with stage I/II endometriosis, 33 women with stage III/IV endometriosis with endometrioma, and 32 women without endometriosis were included in our study.

Interventions: Serum, eutopic endometrial tissues, and/or ectopic endometriotic tissues were collected. Primary eutopic endometrial stromal cells (EuESCs) and ectopic ovarian endometriotic stromal cells (OvESCs) were isolated and cultured from women with ovarian endometrioma, and then treated with or without EGF.

Main Outcome Measures: The concentrations of EGF and hyaluronan in serum were analyzed by enzyme-linked immunoassorbent assay. The expressions and localizations of EGF receptor (EGFR), phosphorylated-(p)EGFR, HAS2, and hyaluronan receptor CD44 in tissues were examined by immunohistochemistry. The mRNA and protein levels of HAS2 in EuESCs and OvESCs were examined by reverse transcription–quantitative polymerase chain reaction (RT-qPCR) and western blot, respectively, and the concentrations of hyaluronan in conditioned medium were examined by enzyme-linked immunoassorbent assay (ELISA). Cell motility was evaluated by transwell migration/invasion assays.

Results: Serum EGF and hyaluronan concentrations were higher in women with stage III/IV endometriosis than in women with stage I/II or without endometriosis. EGFR, pEGFR, HAS2, and CD44 were immunolocalized in eutopic endometrium and ectopic endometriotic lesions, and the expressions of pEGFR and HAS2 were elevated in ectopic endometriotic lesions compared to eutopic endometrium. Treatment with EGF upregulated HAS2 and hyaluronan expression as well as cell migration and invasion in both EuESCs and OvESCs, and pharmaceutical blocking of EGFR abolished these effects. In addition, knockdown of HAS2 by small interfering RNA attenuated both basal and EGF–induced hyaluronan expression and cell motility changes. Notably, ERK1/2 and AKT signaling pathways were shown to be downstream of EGF in regulating HAS2 and hyaluronan expression as well as cell migration and invasion.

Conclusion: EGF increased the expression of endometriosis-associated hyaluronan and its synthase HAS2, both of which mediated EGF–induced stromal cell migration and invasion in women with endometriosis. (Fertil Steril 2020; –: – –. ©2020 by American Society for Reproductive Medicine.)

Key Words: EGF, HAS2, hyaluronan, endometriosis, cell motility

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Endometriosis is a common benign gynecological disease that is characterized by growth and proliferation of endometrial cells at ectopic sites. The ectopic growing endometrial cells share many common characteristics with malignant cells, and these characteristics include cell motility changes such as increased cell migration, invasion, and metastasis (1, 2). Many growth factors and cytokines could modulate extracellular matrix components of endometriotic cells and lead to motility changes of these cells (3).

Epidermal growth factor (EGF) is immunolocalized in both glandular epithelia and stroma of endometriotic tissue, and elevated EGF level is detected in women with endometriosis compare to women without this disease (4, 5). EGF is reported to play a role in endometriosis progression by increasing pro-inflammatory factor matrix metalloproteinase−7 (6). However, little has been known about the role(s) of extracellular matrix components in EGF-mediated endometriosis progression. Hyaluronan, a simple glycosaminoglycan, is an abundant component of extracellular matrix that plays important roles in regulating numerous cellular behaviors such as cell—cell adhesion, proliferation, and differentiation, as well as cell migration and invasion (7, 8). Hyaluronan and its receptor CD44 have been reported to be expressed in endometriotic lesions and may regulate the attachment of endometriotic cells to peritoneal tissues (9, 10). Hyaluronan synthases (HAS), especially HAS2, are critical for synthesis of hyaluronan, as knockout of the Has2 gene in mice poses a developmentally lethal effect (11). HAS2 has been reported to immunolocalized in endometriotic tissues (12) and is associated with cell migration and invasion in certain cancer cells such as colorectal cancers (13).

Interestingly, in epidermal keratinocytes, EGF, via binding to its native receptor EGFR, promotes the expression of HAS2 and contributes to HAS2-mediated hyaluronan production (14). However, in endometriotic tissues, the function of HAS2-hyaluronan system and whether EGF could regulate this system are still unknown.

In this study, we compared the expression levels of EGF, EGFR, HAS2, and hyaluronan in women with or without endometriosis. In addition, we used primary stromal cell cultures from eutopic endometrial and ectopic endometriotic tissues to investigate whether EGF could stimulate HAS2 expression, cell migration, and cell invasion.

**MATERIALS AND METHODS**

**Study Participants**

This study was approved by the Ethics Committee of Women’s Hospital, School of Medicine, Zhejiang University and the Research Ethics Board of the University of British Columbia. All the patients provided informed written consent. A total of 54 women (aged 28–44 years) diagnosed with endometriosis (21 stage I/II early endometriosis and 33 stage III/IV advanced endometriosis with endometrioma according to the revised American Society for Reproductive Medicine [ASRM] classification, proliferative phase) were included in our study. All 33 ovarian endometrioma cases included are classified as stage III/IV. In addition, 32 women (aged 25–42 years) without endometriosis (EuCo, 19 mild hydrosalpinx and 13 unexplained infertility, proliferative phase) were included as endometriosis-free controls. All severe cases of sactosalpinx with the presence of a large amount of fluid or obvious pus were excluded.

None of the patients had been prescribed hormones or other medications known to influence reproductive functions for at least 6 months. Patients with previous hypertension, diabetes mellitus, autoimmune disorder, neoplasm, and other serious medical conditions were excluded from this study. All women had regular menstrual cycles between 25 and 35 days in duration.

**Enzyme-Linked Immunosorbent Assay**

The concentrations of human EGF in serum were quantified using a Human EGF Quantikine enzyme-linked immunosorbent assay (ELISA) Kit (DEG00, R&D Systems) according to the manufacturer’s instructions. Briefly, 96-well polystyrene microplates were coated with monoclonal antibody specific for human EGF. Serum was added to the microplates and incubated at room temperature for 2 hours before sequentially adding horseradish peroxidase–conjugated polyclonal human EGF antibody, substrate solution, and stop solution. The optical density of each well was measured at 450 nm with the correction wavelength set at 540 nm by using a Varioskan Flash plate reader (Thermo Fisher Scientific) after incubation. Similarly, hyaluronan (HA) concentrations (>35 kDa) in the serum and supernatant of cell culture were detected by a Hyaluronan Quantikine ELISA Kit (DHYA0, R&D Systems), and 96-well polystyrene microplates coated with recombinant human Aggrecan were used in this kit.

**Immunohistochemistry and Histoscore Analysis**

Eutopic endometrial tissues from women with and without endometriosis as well as ectopic ovarian endometriotic cysts from women with endometriosis were biopsied or surgically removed before being fixed in formalin and embedded in paraffin. Tissue blocks were cut in approximately 6-μm sections and fixed on glass slides. Sections were deparaffinized, and antigens were exposed using a wet heat epitope retrieval method. Sections were then incubated for 1 hour at room temperature with an anti-EGF receptor (EGFR) antibody (1:200, #4267; Cell Signaling Technology), anti-phosphorylated-EGF receptor (pEGFR) antibody (1:400, #3777; Cell Signaling Technology), anti-HAS2 antibody (1:100, ab140671; Abcam), or anti-CD44 antibody (1:200, #3570; Cell Signaling Technology). EnVision + Dual Link system (Dako) and 3,3′-diaminobenzidine chromogen solution were used to detect the immunoreactivity.

Immunohistochemical scoring (Histoscore) was performed as previously described (15, 16). Briefly, the intensity of immunostaining was classified into four categories (0 = negative, 1 = weak, 2 = moderate, and 3 = strong). The percentage of cells in each specimen with negative, weak, moderate, or strong staining was noted. The sum of the product of the percentage (0–100%) and intensity scores (0–3) were represented as Histoscores. The average Histoscore count of five different fields of each slide at a 200-fold magnification was counted, and all slides were analyzed by two blinded observers.
Experimental Cell Cultures

Eutopic endometria and the endometriotic cysts in proliferative phases were collected from women with endometrioma during laparoscopic procedure. The stage of the menstrual cycle was determined based on the menstrual history, followed by histological confirmation. The method used to isolate eutopic endometrial stromal cells (EuESCs) and ectopic ovarian endometriotic stromal cells (OvESCs) was previously reported by our group (17). In brief, samples were collected, washed with phosphate-buffered saline solution (PBS), and transferred to the laboratory on ice. For EuESCs isolation, eutopic endometrial tissues were washed twice and dissected into fine pieces. For OvESC isolation, endometriotic cysts were rinsed, and blood clots inside the cysts were removed. A sharp blade was used to carefully scrape the interior side of the cyst walls. Debris scraped off from the cyst walls containing the underlying endometrial tissues was collected. Both eutopic endometrial tissues and ovarian endometriotic tissues were digested with type I collagenase (Thermo Fisher Scientific, Waltham, MA) for 60–90 minutes. Remaining debris and epithelial cells were removed by 100- and 40-µm aperture sieves, respectively. Isolated stromal cells were resuspended in phenol red–free Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium containing 10% fetal bovine serum and cultured at 37°C in a humidified 5% CO₂ in air atmosphere. The purity of ESCs was examined by immunostaining for pan-stromal marker vimentin and CD10 (Supplemental Fig. 1). CD10 has been widely used as a biomarker for Eutopic endometrial and endometriotic stromal cells (18, 19).

Only cultures with more than 99% purity were included in our study.

Protein Extraction and Western Blot Analysis

Cells were washed twice in cold PBS prior to being lysed with radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA). Supernatants were collected and protein concentrations were quantified by a Pierce Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) for 60–90 minutes. Equal amounts (50 mg) of protein were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Thermo Fisher Scientific). Membranes were incubated with primary rabbit antibodies against β-actin (1:3000, ab133626; Abcam), HAS2 (1:1000, ab140671; Abcam), ERK1/2 (1:2000, #2117; Cell Signaling Technology, Danvers, MA) and phosphorylated(p)-ERK1/2 (1:2000, #4330; Cell Signaling Technology), AKT (1:2000, #4035; Cell Signaling Technology), and phosphorylated(p)-AKT (1:2000, ab80737; Abcam) at 4°C overnight, followed by horseradish peroxidase–conjugated goat anti-rabbit secondary antibodies (1:2000; Abcam) incubation for 1 hour at room temperature. The signals were detected with an enhanced chemiluminescence detection reagent (Thermo Fisher Scientific), and the bands were quantified by densitometry and analyzed with Quantity One software (Bio-Rad Laboratories).

Reverse transcription—quantitative real-time polymerase chain reaction

For reverse transcription—quantitative real-time polymerase chain reaction (RT-qPCR), RNA was isolated and extracted using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. Reverse transcription was performed using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio, Inc.), and 1 mg of total RNA was reverse transcribed in a 20-µL volume. Real-time polymerase chain reaction was performed with an Applied Biosystems 7900HT system (Applied Biosystems) using SYBR Premix Ex Taq kit (Takara Bio, Inc.). The parameters of real-time PCR were 100 ng of template cDNA, 1 cycle of 95°C for 30 seconds, and 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Specific primers used for amplification were synthesized from Generay (Shanghai, China) with the following sequences: GAPDH (control gene), 5′-ATG GAA ATC CCA TCA CCA TCTT-3′ (forward) and 5′-CGC CCC ACT TGA TTT TGG-3′ (reverse); HAS2, 5′- TTA TGG GCA AAT GTA -3′ (forward) and 5′- ACT TGC TCC AAC GGG TCT -3′ (reverse). The average cycle threshold (Ct) values were calculated from triplicate wells for each sample, and fold changes were determined using 2^ΔΔCt method.

RNA Interference by Small Interfering RNA

RNA interference was performed by small interfering RNA (siRNA) transfection. On-Target plus Non-targeting siRNA #1 (D-001810-01) was purchased from Dharmacon Inc. HAS2 siRNA was synthesized by Genechem Corporation with the following sequence 5′- CCG GGU UCU UCC CUU UTT TT-3′. A quantity of 10⁵ ESCs was seeded in a six-well plate 1 day before transfection. Control siRNA or siRNA against HAS2 was transfected into the cells at a concentration of 30 nM using Lipofectamine RNAiMAX (Life Technologies). Cells were lysed for RT-qPCR or western blot at 48 hours or 72 hours posttransfection, respectively.

Transwell Migration and Invasion Assays

The ESC migration assay was performed in transwell chambers (Corning, Inc. with 8-µm pore size polycarbonate filter inserts, and all experiments were performed using 24-well plates. ESCs were either transfected by siRNA (Ctrl siRNA or siRNA targeting HAS2) for 24 hours or treated by inhibitors (AG1478, U0216, or LY294006) before EGF was added into the culture system for another 24 hours. A quantity of 5 × 10⁴ cells was suspended in 200 µL serum-free DMEM/F12 medium and seeded on the upper chamber. The lower chambers were filled with 500 µL DMEM/F12 containing 10% fetal bovine serum. After 24 hours, cells remaining at the upper chamber were wiped off with a cotton swab, and cells that migrated through the pores and grew on the outer surface of the membrane were fixed and stained with Crystal Violet solution. The stained cells were photographed and counted under a light microscope (100× magnification). Eight fields were counted for each chamber, and triplicate chambers were performed in a single experiment. Results from at least five independent experiments were included for statistical analysis.

Similar procedures with modification were performed for transwell invasion assay. Transwell inserts were precoated with 200 µL of growth factor–reduced Matrigel (0.5 mg/mL,
BD Biosciences) before $1 \times 10^5$ cells were seeded. The incubation time was optimized to 48 hours before cells that invaded through the Matrigel and grew on the outer surface of the membrane were fixed, stained, and counted.

**Statistical Analysis**

Results are presented as the mean ± SEM of at least five independent experiments performed on separate primary cultures, and data were analyzed by GraphPad Prism 8 (GraphPad Software). A parametric Student’s t-test was performed for comparison of two means, and a one-way analysis of variance (ANOVA) followed by a Tukey’s test was used for comparison of multiple means. Values were considered statistically different at $P<.05$, and data sets without a common letter are significantly different.

**RESULTS**

**Serum Levels of EGF and Hyaluronan Are Elevated in Women with Advanced-Stage Endometriosis**

Our ELISA results showed that serum EGF and hyaluronan levels were significantly higher in women with stage III/IV endometriosis compared to women with stage I/II or without endometriosis (Fig. 1A and B). Moreover, the concentrations of hyaluronan are positively correlated with EGF concentrations in the serum of women without (Supplemental Fig. 2A, $R = 0.7211$, $P<.001$), with stage I/II (Supplemental Fig. 2B, $R = 0.8170$, $P<.001$), and with stage III/IV (Supplemental Fig. 2C, $R = 0.8013$, $P<.001$) endometriosis.

**Immunolocalization of HAS2 and CD44, EGFR, and Phosphorylated-(p)EGFR in Eutopic and Ectopic Endometrial Tissues**

EGFR (Fig. 1C–F), pEGFR (Fig. 1G–J), HAS2 (Fig. 1K–N), and CD44 (Fig. 10–R) were immunolocalized at both epithelial and stromal component in eutopic endometrium from women without (EM-free) or with stage I/II (EuEM I/II) or stage III/IV (EuEM III/IV) endometriosis as well as ectopic ovarian endometriotic (OvEM) cyst walls from women with endometrioma. There are no significant differences of the EGFR (Fig. 1S) and CD44 (Fig. 1V) immunostaining intensities among all groups. However, the immunoreactivities of pEGFR in OvEM and EuEM III/IV are significantly higher than those in EM-free and EuEM I/II (Fig. 1T). The immunoreactivity of HAS2 in OvEM is significantly higher than those in EM-free, EuEM I/II, and EuEM III/IV (Fig. 1U).

**EGF Up-Regulates HAS2 and Hyaluronan in Primary Eutopic Endometrial Stromal Cells and Ovarian Endometriotic Stromal Cells**

In eutopic endometrial stromal cells (EuESCs), treatments of 100 ng/mL recombinant human EGF significantly increased HAS2 mRNA levels at 3, 6, 12, and 24 hours (Fig. 2A) as well as protein levels at 6, 12, and 24 hours (Fig. 2B). In addition, stimulation of EuESCs with 100 ng/mL EGF significantly increased the hyaluronan levels in supernatant at 24 hours and 48 hours (Fig. 2C). Treated with higher concentrations (10, 100 ng/mL) of EGF resulted in increased HAS2 mRNA levels at 3 hours (Fig. 2D) and protein levels at 6 hours (Fig. 2E), and the concentration of hyaluronan in supernatant at 48 hours was significantly elevated only by 100 ng/mL EGF (Fig. 2F). Similarly, treatment of ovarian endometriotic stromal cells (OvESCs) with 100 ng/mL EGF up-regulated the mRNA and protein levels of HAS2 as well as the hyaluronan concentrations in supernatant at different time points (Fig. 2G–I). Various concentrations of EGF increased HAS2 mRNA and protein levels as well as hyaluronan secretion in OvESCs (Fig. 2J–L).

**EGFR Is Required for EGF-Induced HAS2 and Hyaluronan Expression as Well as Cell Migration and Invasion**

Pretreatment of EuESCs with AG1478, an ATP-competitive EGFR tyrosine kinase inhibitor, abolished EGF-induced up-regulation of HAS2 mRNA at 3 hours (Fig. 3A), HAS2 protein at 6 hours (Fig. 3B), and hyaluronan secretion at 48 hours (Fig. 3C). In addition, EGF enhanced the migratory (Fig. 3D) and invasive (Fig. 3E) capabilities of EuESCs, and these effects were blocked by pretreatment of AG1478. Similarly, EGF-induced HAS2 mRNA levels at 3 hours (Fig. 3F) and protein levels at 6 hours (Fig. 3G) as well as hyaluronan concentration in supernatant at 48 hours (Fig. 3H) were also abolished by AG1478 in OvESCs. Stimulatory effects of EGF on the migratory (Fig. 3I) and invasive (Fig. 3J) capabilities of OvESCs were also blocked by AG1478.

**HAS2 Mediates EGF-Induced Stromal Cell Migration and Invasion**

Transfection of EuESCs by siHAS2 led to significant reductions of both basal and EGF-induced HAS2 mRNA expressions at 3 hours (Fig. 4A) and protein expressions at 6 hours (Fig. 4B) as well as hyaluronan concentrations in supernatant at 48 h (Fig. 4C). Knockdown of HAS2 by siRNA significantly attenuated the basal migratory (Fig. 4D) and invasive (Fig. 4E) capabilities of EGFR; however, endogenous depletion of HAS2 by siRNA significantly inhibited, but did not completely abolish, EGF-induced EuESC migration (Fig. 4D) and invasion (Fig. 4E). Similar effects were observed in OvESCs (Fig. 4F–H). However, treatment with siRNA significantly attenuated the basal and partially inhibited EGF-induced migratory capabilities (Fig. 4I), while completely abolishing both basal and EGF-induced invasiveness (Fig. 4J) of OvESCs.

**ERK1/2 and AKT Signaling Pathways Are Involved in EGF-Enduced HAS2 and Hyaluronan Expression**

In OvESCs, treatment of 100 ng/mL EGF significantly increased ERK1/2 and AKT phosphorylation at 5, 10, 30, 60, and 180 minutes (Supplemental Fig. 3A), and these effects could be completely abolished by pretreatment of U0126 and LY294002 (Supplemental Fig. 3B). Pretreatment of OvESCs by U0126 or LY294002 significantly attenuated both basal and EGF-induced HAS2 mRNA expression at 3 hours (Supplemental Fig. 3C) and protein expression at 6 hours (Supplemental Fig. 3D) as well as the concentrations
Expressions of EGF, hyaluronan, EGFR, phosphorylated-(p)EGFR, HAS2, and CD44 in women with or without endometriosis. Concentrations of (A) EGF and (B) hyaluronan in the serum of women without endometriosis (EM-free), with early stages of endometriosis (EM I/II) and with advanced stages of endometriosis (EM III/IV) were examined by enzyme-linked immunosorbent assay and are represented by dot plots. (C–F) Immunolocalizations of EGFR, (G–J) pEGFR, (K–N) HAS2, and (O–R) CD44 in eutopic endometrium from endometriosis-free women (EM-free), eutopic endometrium from women with early stages of endometriosis (EuEM I/II) and advanced stages of endometriosis (EuEM III/IV), and ectopic ovarian endometriotic (OvEM) cyst walls from women with advanced stages of endometrioma are presented. Immunoreactivity intensities of (S) EGFR, (T) pEGFR, (U) HAS2, and (V) CD44 were evaluated by Histoscore and are represented by dot plots. Data were analyzed by one-way analyses of variance followed by Tukey’s post hoc tests. Results are presented as mean ± SEM; values without a common letter are significantly different (P<.05). EGF = epidermal growth factor; EGFR = EGF receptor; EM = endometriosis; HAS2 = hyaluronan synthase 2; SEM = standard error of the mean.

FIG. 2

EGF upregulates HAS2 and hyaluronan expressions in both eutopic endometrial stromal cells (EuESCs) and ovarian endometriotic stromal cells (OvESCs) from patients with endometriosis. (A – C) EuESCs or (G–I) OvESCs were treated with vehicle control (Ctrl) or 100 ng/mL EGF; then the (A, G) mRNA and (B, H) protein levels of HAS2 were analyzed at different time-points by RT-qPCR and western blot, respectively, and (C, I) hyaluronan concentrations in supernatant were examined by ELISA. (D – F) EuESCs or (J–L) OvESCs were treated with increasing concentrations of EGF (1, 10, 100 ng/mL), and (D, J) HAS2 mRNA levels were analyzed after 3 hours by RT-qPCR, (E, K) protein levels were detected after 6 hours by western blot, and (F, L) hyaluronan levels in supernatant were examined after 48 hours by ELISA. Data were analyzed by one-way analysis of variance followed by Tukey’s post hoc tests. Results are expressed as the mean ± SEM from at least five primary cell cultures in each experiment. Values without a common letter are significantly different (P < 0.05). EGF = epidermal growth factor; EGFR = EGF receptor; ELISA = enzyme-linked immunosorbent assay; EM = endometriosis; HAS2 = hyaluronan synthase 2; RT-qPCR = reverse transcription–quantitative polymerase chain reaction; SEM = standard error of the mean.

FIG. 3

EGFR mediates EGF-induced HAS2 and hyaluronan expression as well as cell migration and invasion. (A – C) EuESCs or (F – H) OvESCs were pretreated with 10 μM AG1478 for 1 hour before 100 ng/mL EGF treatment, and (A, F) HAS2 mRNA levels were analyzed by RT-qPCR after 3 hours, (B, G) HAS2 protein levels were detected after 6 hours by western blot, and (C, H) hyaluronan concentrations were examined after 48 hours by ELISA. (D, E) EuESCs or (I, J) OvESCs were pretreated with 10 μM AG1478 for 1 hour, then cells were incubated in the polycarbonate filter inserts with 100 ng/mL EGF for 24 hours to (D, I) examine migratory capability and for 48 hours to (E, J) examine invasiveness. Data were analyzed by one-way analysis of variance followed by Tukey’s post hoc tests. Results are expressed as the mean ± SEM from at least five primary cell cultures in each experiment. Values without a common letter are significantly different (P < .05). EGF = epidermal growth factor; EGFR = EGF receptor; ELISA = enzyme-linked immunosorbent assay; EM = endometriosis; EuESCs = eutopic endometrial stromal cells; HAS2 = hyaluronan synthase 2; OvESCs = ovarian endometriotic stromal cells; RT-qPCR = reverse transcription–quantitative polymerase chain reaction; SEM = standard error of the mean.

HAS2 is involved in EGF-induced hyaluronan secretion as well as cell migration and invasion. (A–C) EuESCs or (F–H) OvESCs were transfected with 25 nM control siRNA (siCtrl) or HAS2 siRNA (siHAS2) for 48 hours before treatment with 100 ng/mL EGF. (A, F) HAS2 mRNA levels were analyzed after 3 hours by RT-qPCR, (B, G) HAS2 protein levels were detected after 6 hours by western blot, and (C, H) hyaluronan concentrations were examined after 48 hours by ELISA. Cells were transfected with 25 nM siCtrl or siHAS2 for 48 hours, then cells were incubated in the polycarbonate filter inserts with 100 ng/mL EGF for 24 hours to (D, I) examine migratory capabilities assay, and for 48 hours to (E, J) evaluate invasiveness. Data were analyzed by one-way analysis of variance followed by Tukey’s post hoc tests. Results are presented as the mean ± SEM from at least five primary cell cultures in each experiment. Values without a common letter are significantly different (P < 0.05). EGF = epidermal growth factor; EGFR = EGF receptor; ELISA = enzyme-linked immunosorbent assay; EM = endometriosis; HAS2 = hyaluronan synthase 2; RT-qPCR = reverse transcription–quantitative polymerase chain reaction; SEM = standard error of the mean.

of hyaluronan in supernatant at 48 h (Supplemental Fig. 3E). Furthermore, U0126 attenuated basal and abolished EGF-induced OvESC migration (Supplemental Fig. 3F) and invasion (Supplemental Fig. 3G), whereas LY294002 attenuated basal and partially inhibited EGF-induced OvESC migration (Supplemental Fig. 3F) and invasion (Supplemental Fig. 3G).

DISCUSSION

It has long been known that EGF is locally produced by both epithelial and stromal components of endometriotic lesions (4); however, there has been some debate about whether the expression of EGF in eutopic and/or ectopic tissues is involved in the pathogenesis of endometriosis. For example, an early report suggested that the protein levels of EGF in peritoneal fluid of women with or without endometriosis were highly variable, and no significant differences were found between these two groups of women (20). In contrast, in our study, we found that the serum concentrations of EGF were significantly elevated in women with advanced stages (III/IV) of endometriosis compared to those in women with early stages or without endometriosis. In agreement with our results, a recent article reported increased EGF immunoreactivities in advanced stages of ovarian endometrioma compare to those in early stages (6), whereas the above early report included mostly (37 of 41) participants with early stages of endometriosis. The large number of participants with early stages of endometriosis in that study might be the reason for the lack of statistical differences, as in our study there were also no significant differences in EGF expression between women without and with early stages of disease (Fig. 1A). Collective findings from our results and all of the above studies revealed an interesting finding, namely, that EGF might be involved in the disease progression of advanced but not early stages of endometriosis.

Thus far, little is known about the immunolocalization of hyaluronan synthase in endometriosis. A recent study has reported that HAS1, 2, and 3 are immunolocalized in the epithelial and stromal cells isolated from the menses of women with or without endometriosis, whereas the expression of HAS in ectopic endometriotic implants was not revealed by this study (12). To our knowledge, our study was the first to report that HAS2 was immunolocalized in both the epithelial and stromal components of ovarian endometrioma. In addition, we found that the immunoreactivity of HAS2 was elevated in ovarian endometrioma lesions, which might indicate a role of HAS2 in the disease progression of endometriosis. Notably, previous studies have shown that the HAS and hyaluronan were readily detected in endometrial cancer, and elevated hyaluronan levels were associated with intensive myometrial invasion of endometrial cancer (21, 22). In addition, the levels of hyaluronan degradation enzyme hyaluronidases were found to be decreased in endometrial cancer, leading to the accumulation of hyaluronan (23). Collectively, the contribution of hyaluronan in advanced stages of malignant endometrial cancer was in agreement with our study in benign conditions, as increased hyaluronan levels was associated with advanced stages of endometriosis. The mechanism(s) about how hyaluronan contributes to invasion and metastasis of benign and malignant endometrial cells warrants further investigation.

No significant differences of the hyaluronan receptor CD44 immunoreactivities were detected between ectopic and eutopic endometrium in women, with or without endometriosis, according to our study. Other mechanism(s) rather than expression change of CD44 may be involved in hyaluronan-associated endometriosis. Alternative mRNA splicing may be a potential mechanism, as it has been found that menstrual endometrial cells from women with endometriosis express increased mRNA levels of CD44 splice variants (24). In addition, alternation of glycosylation of CD44 may also contribute to endometriosis, as inhibition of O- and N-glycosylated CD44 in endometrial stromal cells attenuates their capability of attachment to peritoneal mesothelial cells (25). Moreover, higher soluble forms of CD44 were found in peritoneal fluid of women with endometriosis compared to women without endometriosis (26). This phenomenon may suggest the body’s compensatory defense mechanism against endometriosis, as the soluble form of CD44, a competitive inhibitor of hyaluronan, may reduce the capability of endometriotic cell attachment to the peritoneal cavity.

Interestingly, in our study, we found a constant trend that the same dosage of EGF exerted stronger stimulatory effects in OvESCs compared to those in EuESCs. For example, on average, an approximate threefold increase in HAS2 mRNA levels was induced by 100 ng/mL EGF at 3 hours in EuESCs, whereas an eightfold elevation of HAS2 mRNA levels was induced by the same dosage of EGF at 3 hours in OvESCs (Fig. 2). A similar effect could also be observed in EGF-induced HAS2 protein expression, hyaluronan secretion, as well as cell migration and invasion. Dysregulated EGFR and its downstream signaling in endometriotic cells may be a possible explanation of this phenomenon. Based on our immunohistochemistry results, no significant differences in EGFR immunoreactivity were observed between eutopic endometrial and ectopic endometriotic tissues from women with endometrioma (Fig. 1), and a similar finding in a previous study was also reported (27). However, the immunoreactivity of phosphorylated EGFR (p-EGFR) in ovarian endometrioma is significantly increased compared to that of early-stage (I/II) eutopic endometrium with a borderline increase compared to that of advanced-stage (III/IV) eutopic endometrium (Fig. 1). A polymorphism at position 2073 of the EGFR gene was reported to be associated with susceptibility of endometriosis (28), and this polymorphism may associate with altered EGFR function and/or activity leading to downstream change of effect.

Our data showed that EGF could promote HAS2 expression, hyaluronan secretion, as well as cell migration and invasion in endometrial and endometriotic stromal cells. In agreement with our findings, a previous study in epidermal keratinocytes showed increases of HAS2 mRNA levels, total cell-associated as well as intracellular hyaluronan, and cell migratory capability, but not proliferation, with the administration of EGF (14). In addition, we have found that MEK/ERK1/2 and PI3K/AKT pathways were involved in EGF-induced HAS2 expression and cell motility changes. In supportive of our finding, other authors reported that EGF-stimulated HAS2 expression in ovarian carcinoma may be mediated by MEK/ERK1/2 signaling (29). In addition, the interaction of EGFR and hyaluronan receptor
CD44 may play an important role in triggering downstream MEK/ERK1/2 signaling (30). To our knowledge, we are the first to report the direct involvement of PI3K/AKT signaling pathway in EGF-induced HAS2 expression and hyaluronic secretion in any cell type, and adding a new pathway to the existing signaling framework may help us to further appreciate the complexity of endocrine regulation of extracellular matrix secretion.

In conclusion, we have shown that expression of EGF, hyaluronan, and HAS2 are elevated in women with endometriosis. In addition, we have demonstrated that EGF could directly stimulate HAS2 expression, hyaluronan secretion, as well as cell migration and invasion in eutopic endometrial and ectopic endometriotic stromal cells from women with ovarian endometrioma, and these effects are mediated by EGFR as well as MEK/ERK1/2 and PI3K/AKT signaling pathways. Our findings may provide insight into the possible role(s) of EGF-EGFR and HAS2-hyaluronan systems in endometriosis disease progression.

Acknowledgements: This work was supported by National Science Foundation of China (No. 81771550, No. 81671426, and No. 81873825)

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